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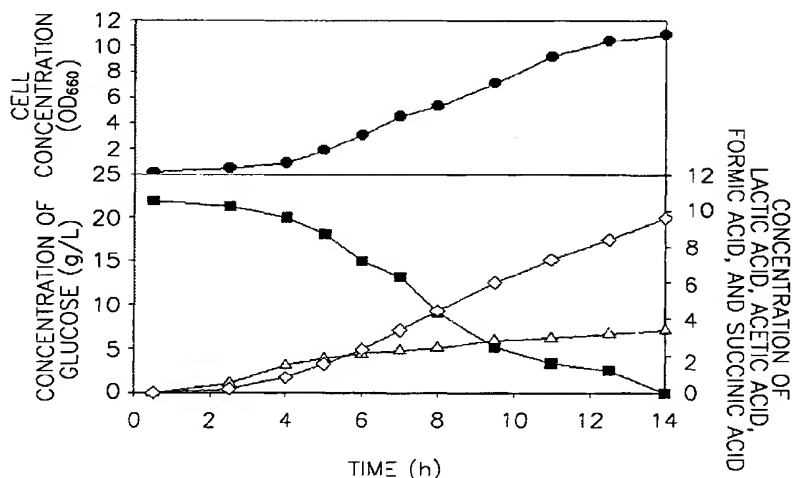
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(54) Title: ORGANIC ACID PRODUCING MICROORGANISM AND PROCESS FOR PREPARING ORGANIC ACIDS EMPLOYING THE SAME



(57) Abstract: A novel microorganism capable of producing organic acids, *Mannheimia* sp. 55E, and a process for producing organic acid through anaerobic and aerobic incubation using the novel microorganism are provided. The method of producing an organic acid using the microorganism involves incubating *Mannheimia* sp. 55E with Accession Number KCTC 0769BP in a medium under anaerobic or aerobic conditions and obtaining an organic acid from the medium. *Mannheimia* sp. 55E produces succinic acid, lactic acid, and formic acid under anaerobic conditions saturated with CO₂, and succinic acid, lactic acid, acetic acid, and formic acid under aerobic or N₂ anaerobic conditions. *Mannheimia* sp. 55E is a facultative anaerobe tolerant of oxygen. Thus, the use of *Mannheimia* sp. 55E in producing organic acids can eliminate a problem of process instability, which would occur by the presence of oxygen in a fermentation process of producing organic acids using an obligate anaerobic microorganism.



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**ORGANIC ACID PRODUCING MICROORGANISM AND
PROCESS FOR PREPARING ORGANIC ACIDS EMPLOYING
THE SAME**

5 Technical Field

 The present invention relates to a microorganism capable of producing organic acids and a process for producing organic acids using the microorganism, and more particularly, to a new microorganism, *Mannheimia* sp. 55E, capable of producing organic acids and a process
10 for producing organic acids through incubation of the microorganism in anaerobic or aerobic conditions.

Background Art

 Thanks to the progress of the petrochemical industry, most
15 chemical substances have been produced from petroleum and natural gases. However, restrictions on the production of chemical substances is becoming more rigid worldwide with the recent rise of environmental concerns such as global warming, thereby increasing the need for the development of an alternate to the conventional process of producing
20 chemicals.

 The rapid advance of microorganism cultivation and genetic engineering technology has placed biochemical productions of chemical substances at a competitive position to the conventional petrochemical process. Biochemical methods are known to be environmentally
25 compatible, thus being capable of solving environmental problems, because they can suppress generation of gases that cause global warming, such as carbon dioxide (CO₂), with use of inexpensive renewable resources. Therefore, approaches to cost reduction through isolation of new strains and process improvement are getting spread in

the biochemical field. In addition, increased market values of biochemical materials have boosted research into the production of the biochemical materials from biomass using microorganisms throughout the world.

5 In particular, focus on biochemical materials, particularly succinic acid and lactic acid, is increasing due to their cost competitiveness and wide applications as biodegradable plastic monomers, food additives, and intermediate products of other organic compounds.

A study on the biological production of succinic acid was started
10 in 1938 by *Lockwood* who reported production of succinic acid from sugar using *Fusarium martii* with a 18% yield. It was reported that production of succinic acid through glycolysis of a variety of anaerobic microorganisms such as *Succinivibrio dextrinosolvens*, *Fibrobacter succinogenes*, and *Ruminococcus flavefaciens* (Zeikus, Annu. Rev.
15 Microbiol., 34:423-464,1980). Any microorganism capable of producing succinic acid with high yield for industrial use, except for *Anaerobiospirillum succiniciproducens* known to be able to produce succinic acid from glucose with high yield and concentration in the presence of excess carbon, was not disclosed (David et al., *Int. J. Syst.*
20 *Bacteriol.*, 26:498-504, 1976). Because *Anerobiospirillum succiniciproducens* is an obligate anaerobic microorganism, stable fermentation using the microorganism for production of the succinic acid is not ensured even with the presence of trace oxygen.

A variety of factors, such as production yield, fermentation
25 conditions, etc., should be carefully considered for economical production of organic acids through microorganism fermentation. In particular, isolation of new microorganisms capable of producing organic acids has priority over the other factors to consider. Most microorganisms currently available to produce organic acids are
30 anaerobic microorganisms, so costly cultivation equipment is required,

and it is very likely that intended fermentation fails due to the presence of trace oxygen. Therefore, there is an urgent need for development of microorganisms having a tolerance to oxygen and capable of producing organic acids with high yield.

5 As a result of research on such oxygen-tolerant microorganisms being able to produce organic acids with high throughput, the present inventors have succeeded in separating a new facultative anaerobe, *Mannheimia* sp. 55E, from the bovine rumen fluid, and identifying the new microorganism to be tolerant of oxygen and able to produce organic
10 acids in anaerobic and aerobic conditions.

Disclosure of the Invention

It is a first object of the present invention to provide a new microorganism, *Mannheimia* sp. 55E (KCTC 0769BP), capable of
15 producing organic acids.

It is a second object of the present invention to provide a process for producing organic acids under anaerobic and aerobic conditions using the microorganism.

20 Brief Description of the Drawings

FIG. 1 is a phylogenetic tree constructed by 16S RNA analysis for *Mannheimia* sp. 55E;

FIG. 2 is a scanning electron microscope (SEM) photo showing the morphological characteristics of *Mannheimia* sp. 55E;

25 FIG. 3 is a graph showing the fermentation characteristics of *Mannheimia* sp. 55E under anaerobic conditions saturated with CO₂;

FIG. 4 is a graph showing the fermentation characteristics of *Mannheimia* sp. 55E under anaerobic conditions saturated with N₂; and

FIG. 5 is a graph showing the fermentation characteristics of
30 *Mannheimia* sp. 55E under aerobic conditions.

Best mode for carrying out the Invention

To isolate a new microorganism tolerant of oxygen and capable of producing organic acids with high yield, the present inventors inoculated dilute samples from the rumen fluid on a solid medium to give rise to individual colonies and incubated on a growth medium containing excess carbon dioxide (CO₂) to screen strains having the ability to produce succinic acid. The screened strain was subjected to 6S rDNA analysis to determine whether they have similarity to previously known strains. As a result, the isolated strain was identified as a new microorganism with a 95% similarity to *Mannheimia varigena* and designated as *Mannheimia* sp. 55E. A deposit of *Mannheimia* sp. 55E was made April 10, 2000 to the Korean Collection for Type Cultures (KCTC), an International Depository Authority (IDA) in Korea Research Institute of Bioscience and Biotechnology (KRIBB) (52 Oun-dong, Yusong-gu, Taejon, Republic of Korea) and was given Accession Number KCTC 0769BP.

Mannheimia sp. 55E identified in the present invention is facultative anaerobic, gram-negative, non-motile, appears as rods or coccobacilli, does not form endospores, and has the ability to produce organic acids such as succinic acid, lactic acid, acetic acid, formic acid, etc. *Mannheimia* sp. 55E has a growth temperature range of 30-50°C with an optimal growth temperature of 39°C and a pH range of 6.0-7.5 with an optimal pH of 6.5.

A process for producing organic acids using *Mannheimia* sp. 55E involves incubating *Mannheimia* sp. 55E (KCTC 0769BP) under anaerobic or aerobic conditions and obtaining organic acids therefrom. The anaerobic conditions are created with a supply of carbon dioxide (CO₂) or nitrogen at a flow rate of 0.1-0.4 vvm, preferably 0.2-0.3 vvm, and most preferably 2.25 vvm. The aerobic conditions are created with

a supply of oxygen (O₂) at a flow rate of 0.5-1.0 vvm, preferably 0.7-0.8 vvm, and most preferably 0.75 vvm.

Any medium is available for the culture of *Mannheimia* sp. 55E without limitations, but preferably the content of glucose in a medium is
5 in the range of 5-60 g/L, and most preferably 20 g/L. The incubation is carried out at a temperature of 35-45°C, preferably at a temperature of 38-41°C, and most preferably at 39°C, and at pH 6.0-7.5, preferably at pH 6.3-6.7, and most preferably at pH 6.5.

Lactic acid, citric acid, succinic acid, or formic acid is produced
10 under the anaerobic conditions. It is preferable to produce the anaerobic conditions using CO₂ for higher yield of succinic acid and N₂ for higher yield of lactic acid. Lactic acid, citric acid, or formic acid is produced under the aerobic conditions with the lactic acid having the highest yield.

15

The present invention will be described in greater detail by means of the following examples. The following examples are for illustrative purposes and are not intended to limit the scope of the invention.

20 Example 1: Isolation and Identification of *Mannheimia* sp. 55E by 16S rDNA Sequence Analysis

1 ml of the bovine rumen fluid was subjected to 100 fold dilution with a medium consisting of 20 g of glucose, 5 g of polypeptone, 5 g of yeast extract, 3 g of K₂HPO₄, 1 g of NaCl, 5 g of (NH₄)₂SO₄, 0.2 g of
25 CaCl₂·2H₂O, 0.2 g of MgCl₂·6H₂O, and 5 g of Na₂CO₃ per liter, followed by plating on a 1%-agarose solid medium having the same composition as the medium above and incubation for 10 hours. Individual colonies were isolated from the cultures and transferred to and incubated in 3 ml of the above medium saturated with CO₂ for 10 hours. The yield of

succinic acid in the medium was measured by high performance liquid chromatography (HPLC, Aminex HPX-87H column, Bio-Rad, U.S.A.), and a strain producing succinic acid with a 50% or greater yield was screened. As a result of a physiological property assessment on the
5 screened strain, the screened strain was found to be gram negative and non-endospore forming. The strain growth was observed at a temperature of 30-50°C with the optimal growth temperature of 39°C, and at a pH of 6.0-7.5 with the optimal growth pH of 6.5.

Genomic DNA was isolated from the screened strain by applying
10 the Rochelle's method (Rochelle et al., FEMS Microbiol., Lett., 100:59-66, 1992). Polymerase chain reaction (PCR) was carried out using the isolated genomic DNA as a template, primer 1 (5'-AGAGTTTGATCMTGGCTCAG-3' (SEQ ID NO. 1)), and primer 2 (5'-AAGGAGGTGWTCCARCC-3' (SEQ ID NO. 2)) to amplify 16S rDNA.
15 The sequence of the amplified products of 16S rDNA was identified, and the similarity of the 16S rDNA to other known strains was analyzed using a DNA sequence analysis program, PHYLIP (PHYlogeny Inference Package, version 3.5c). Table 1 shows the result of the similarity analysis for species, and FIG. 1 is a phylogenetic tree constructed
20 through the similarity analysis.

As shown in Table 1 and FIG. 1, the isolated strain is proved to be a new strain that belongs to the *Mannheimia* genus in the *Pasteurellaceae* family with a 95.1% similarity to *Mannheimia variaena*, and designated *Mannheimia* sp. 55E. A deposit of *Mannheimia* sp. 55E
25 was made April 10, 2000 to the Korean Collection for Type Cultures (KCTC), an International Depository Authority (IDA) in Korea Research Institute of Bioscience and Biotechnology (KRIBB) (52 Oun-dong, Yusong-gu, Taejon, Republic of Korea) and was given Accession Number KCTC 0769BP.

Table 1. Results of 16S r DNA Analysis for Similarity

Species	Code No.	Similarity
<i>Mannheimia varigena</i> (CCUG 38462T)	AF053893	95.1
<i>Mannheimia granulomatis</i> (ATCC 49244T)	AF053902	94.9
<i>Mannheimia ruminantis</i> (CCUG 38470T)	AF053900	94.7
<i>Mannheimia glucosida</i> (CCUG 38457T)	AF053889	94.7
<i>Pasteurella trehalosi</i> (NCTC 11550)	U57073	94.7
<i>Pasteurella dagmatis</i> (ATCC 43325T)	M75051	94.3
<i>Mannheimia haemolytica</i> (NCTC 9380T)	AF060699	94.1
<i>Pasteurella avium</i> (NCTC 11297T)	M75058	94.0
<i>Pasteurella aerogenes</i> (ATCC 27883T)	M75048	94.0
<i>Pasteurella bettyae</i> (CCUG 2042T)	L06088	94.0
<i>Pasteurella volantium</i> (NCTC 3438T)	M75070	93.8
<i>Pasteurella mairii</i> (CCUG 27189T)	AF024532	93.8
<i>Pasteurella multocida</i> (NCTC 10322T)	M35018	93.8
<i>Pasteurella langaaensis</i> (ATCC 43328T)	M75053	93.7
<i>Pasteurella gallinarum</i> (NCTC 11188T)	M75059	93.5
<i>Pasteurella stomatis</i> (ATCC 43327T)	M75050	93.2
<i>Pasteurella canis</i> (ATCC 43326T)	M75049	93.1
<i>Haemophilus influenzae</i> T	M35019	92.5
<i>Pasteurella pneumotropica</i> (NCTC 8141T)	M75083	92.3
<i>Pasteurella anatis</i> (ATCC 43329T)	M75054	92.0
<i>Pasteurella testudinis</i> (CCUG 19802T)	L06090	91.3
<i>Vibrio cholerae</i> (ATCC 14035T)	Z21856	87.8
<i>E. coli</i>	J01695	86.6

Example 2: Characteristics of *Mannheimia* sp. 55E

- 5 *Mannheimia* sp. 55E was cultured on a blood agar plate, which is known to be suitable for the growth of *Mannheimia* species, at 39°C for 24 hours. As a result, colonies having a diameter of 1-2 mm with smooth greyish surface were observed. Single cells of *Mannheimia* sp. 55E were observed using a scanning electron microscope (SEM). As

shown in FIG. 2, cells of *Mannheimia* sp. 55E appear as rods or coccobaccilli having dimensions 0.3-0.5 by 1.0 microns.

Example 3: Fermentation Characteristics of *Mannheimia* sp. 55E
5 under anaerobic conditions saturated with CO₂

To investigate fermentation power of *Mannheimia* sp. 55E, it was incubated under anaerobic conditions saturated with CO₂, and the reaction products were analyzed.

100 ml of a pre-culture medium consisting of 20 g of glucose, 5 g
10 of polypeptone, 5 g of yeast extract, 3 g of K₂HPO₄, 1 g of NaCl, 1 g of (NH₄)₂SO₄, 0.2 g of CaCl₂·2H₂O, 0.2 g of MaCl₂·6H₂O, and 10 g of MgCO₃ per liter was prepared, and CO₂ gas was supplied to the medium.

Inoculation of *Mannheimia* sp. 55E was followed by pre-culture at 39°C for 9 hours.

15 0.9 ml of a main culture medium consisting of 20 g of glucose, 5 g of polypeptone, 5 g of yeast extract, 3 g of K₂HPO₄, 1 g of NaCl, 1 g of (NH₄)₂SO₄, 0.2 g of CaCl₂·2H₂O, 0.2 g of MaCl₂·6H₂O, and 10 g of MgCO₃ per liter was placed inside a 2.5L incubator. 100 ml of the pre-culture of succinic acid producing microorganism was transferred to
20 the main culture medium, followed by fed-batch culture at 39°C at pH 6.5 with supply of CO₂ gas at a flow rate of 0.25 vvm. Culture media were sampled at 1-hour intervals during the incubation and cell concentration, and the amounts of succinic acid, glucose, acetic acid, and formic acid were measured by a spectrophotometer (Ultraspec 3000, Pharmacia
25 Biotech., Sweden).

FIG. 3 is a graph showing variations in concentration of cells (●), succinic acid (○), glucose (■), lactic acid (◇), and formic acid (□) with respect to incubation time. As shown in FIG. 3, after a 7.5-hour incubation, 19.8 g/L of glucose is consumed, and 13.3 g/L lactic acid is

produced with a yield of 67% (amount of produced succinic acid per amount of consumed glucose). Volumetric productivity for succinic acid (amount of produced succinic acid per hour) is 1.8 g/L/h.

The method of producing succinic acid using *Mannheimia* sp. 55E
5 under anaerobic conditions saturated with CO₂ has a slightly lower yield, but about 80% greater volumetric productivity than a conventional anaerobic method of producing succinic acid using *Anaerobiospirillum succiniciproducens* (ATCC 29305) (Lee et al., *Enzyme Microbiol. Technol.*, 24:549-554, 1999).

10

Example 4: Fermentation Characteristics of *Mannheimia* sp. 55E under anaerobic conditions saturated with N₂

To investigate fermentation power of *Mannheimia* sp. 55E under anaerobic conditions saturated with N₂, incubation was carried out for 22
15 hours in the same manner as in Example 3, except that nitrogen was supplied at a flow rate of 0.25 vvm. Culture media were sampled at 1-hour intervals during the incubation, and cell concentration and the amounts of lactic acid, glucose, formic acid, acetic acid, and succinic acid were measured by the same method as in Example 3.

20 FIG. 4 is a graph showing variations in concentration of cells (●), succinic acid (○), lactic acid (◇), glucose (■), formic acid (□), and acetic acid (Δ) with respect to incubation time. Unlike the result of FIG. 3, as shown in FIG. 4, *Mannheimia* sp. 55E according to the present invention produces lactic acid as a major product and succinic acid with a low yield
25 less than 9% under anaerobic conditions saturated with N₂. During the anaerobic fermentation, 18 g/L of glucose is consumed, and 9.8 g/L lactic acid is produced with a yield of 54% and a volumetric productivity of 0.45 g/L/h.

This result indicates that *Mannheimia* sp. 55E needs CO₂ to
30 produce succinic acid. *Mannheimia* sp. 55E is considered as fixing CO₂

to use it in the production of succinic acid.

Example 5: Fermentation Characteristics of *Mannheimia* sp. 55E under aerobic conditions saturated with O₂

5 To investigate fermentation power of *Mannheimia* sp. 55E under aerobic conditions, incubation was carried out for 14 hours in the same manner as in Example 3, except that oxygen was supplied at a flow rate of 0.75 vvm. Culture media were sampled at 1-hour intervals during the incubation, and cell concentration and the amounts of lactic acid,
10 glucose, and acetic acid were measured by the same method as in Example 3.

FIG. 5 is a graph showing variations in concentration of cells (●), lactic acid (◇), glucose (■), and acetic acid (Δ) with respect to incubation time. As shown in FIG. 5, *Mannheimia* sp. 55E according to the present
15 invention shows an increase in cell concentration up to 11 by optical density (OD) at 660 nm after fermentation under O₂ aerobic conditions. This result indicates that *Mannheimia* sp. 55E is a facultative anaerobe whose cell growth is unaffected by the presence of oxygen. This aerobic fermentation using *Mannheimia* sp. 55E does not produce
20 succinic acid and formic acid at all and produces lactic acid as a major product. During the aerobic fermentation, 22 g/L of glucose is consumed, and 9.8 g/L lactic acid is produced with a yield of 45% and a volumetric productivity of 0.7 g/L/h.

As supported by the results described above, since *Mannheimia*
25 sp. 55E according to the present invention is a facultative anaerobe, removal of dissolved oxygen is unnecessary for the production of succinic acid under CO₂ anaerobic conditions. It is expected that the production of succinic acid be unaffected by the presence of oxygen, as long as the amount of CO₂ supplied for the fermentation is sufficient
30 enough.

Industrial Applicability


The present invention provides *Mannheimia* sp. 55E, a novel microorganism capable of producing organic acids, and a process for
5 producing organic acid through anaerobic and aerobic fermentation using the novel microorganism. As described above, *Mannheimia* sp. 55E according to the present invention produces succinic acid, lactic acid, and formic acid under CO₂ anaerobic conditions, and succinic acid, lactic acid, acetic acid, and formic acid under aerobic or N₂ anaerobic
10 conditions. *Mannheimia* sp. 55E according to the present invention is a facultative anaerobe tolerant of oxygen. Thus, the use of *Mannheimia* sp. 55E in producing organic acids can eliminate a problem of process instability, which would occur by the presence of oxygen in the conventional fermentation process of producing organic acids using an
15 obligate anaerobic microorganism.

While this invention has been particularly shown and described with reference to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made t herein without departing from the spirit and scope of the
20 invention as defined by the appended claims.

**Indications Relating to Deposited Microorganism(s) or
Other Biological Material**
(PCT Rule 13 bis)

5

A. The indications made below relate to the deposited microorganism(s) or other biological material referred to in the description on page 6, line 26	
B. Identification of Deposit Additional deposits will be described on the following separate page <input type="checkbox"/>	
Name of depositary Institution Korean Collection for Type Cultures	
Date of deposit 10 April 2000	Accession Number KCTC 0769BP
C. Additional Indications	
D. Particular Facts in the Indications	
E. Separate Supplementary Indications	

For Receiving Office Use Only
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PCT/RO/134(July 1998)

What is claimed is:

1. *Mannheimia* sp. 55E with Accession Number KCTC 0769BP, capable of producing an organic acid under anaerobic and aerobic conditions.

5

2. A process for producing an organic acid, the process comprising:

incubating *Mannheimia* sp. 55E with Accession Number KCTC 0769BP of claim 1 in a medium containing 5-60 g/L of glucose under
10 anaerobic conditions saturated with carbon dioxide or nitrogen; and
obtaining an organic acid from the medium.

3. The process of claim 2, wherein the organic acid is one of succinic acid, formic acid, acetic acid, and lactic acid.

15

4. The process of claim 2, wherein the incubation is carried out at a temperature of 35-45°C at a pH of 6.0-7.5 with a supply of carbon dioxide or nitrogen at a flow rate of 0.1-0.4 vvm.

20

5. A process for producing succinic acid, the process comprising:

incubating *Mannheimia* sp. 55E with Accession Number KCTC 0769BP of claim 1 in a medium containing 5-60 g/L of glucose at a temperature of 35-45°C at a pH of 6.0-7.5 with a supply of carbon
25 dioxide at a flow rate of 0.1-0.4 vvm; and
obtaining succinic acid from the medium.

6. A process for producing lactic acid, the process comprising:

30 incubating *Mannheimia* sp. 55E with Accession Number KCTC

0769BP of claim 1 in a medium containing 5-60 g/L of glucose at a temperature of 35-45°C at a pH of 6.0-7.5 with a supply of nitrogen at a flow rate of 0.1-0.4 vvm; and
obtaining lactic acid from the medium.

5

7. A process for producing an organic acid, the process comprising:

incubating *Mannheimia* sp. 55E with Accession Number KCTC 0769BP of claim 1 in a medium containing 5-60 g/L of glucose under
10 aerobic conditions; and
obtaining an organic acid from the medium.

8. The process of claim 7, wherein the organic acid is one of lactic acid and acetic acid.

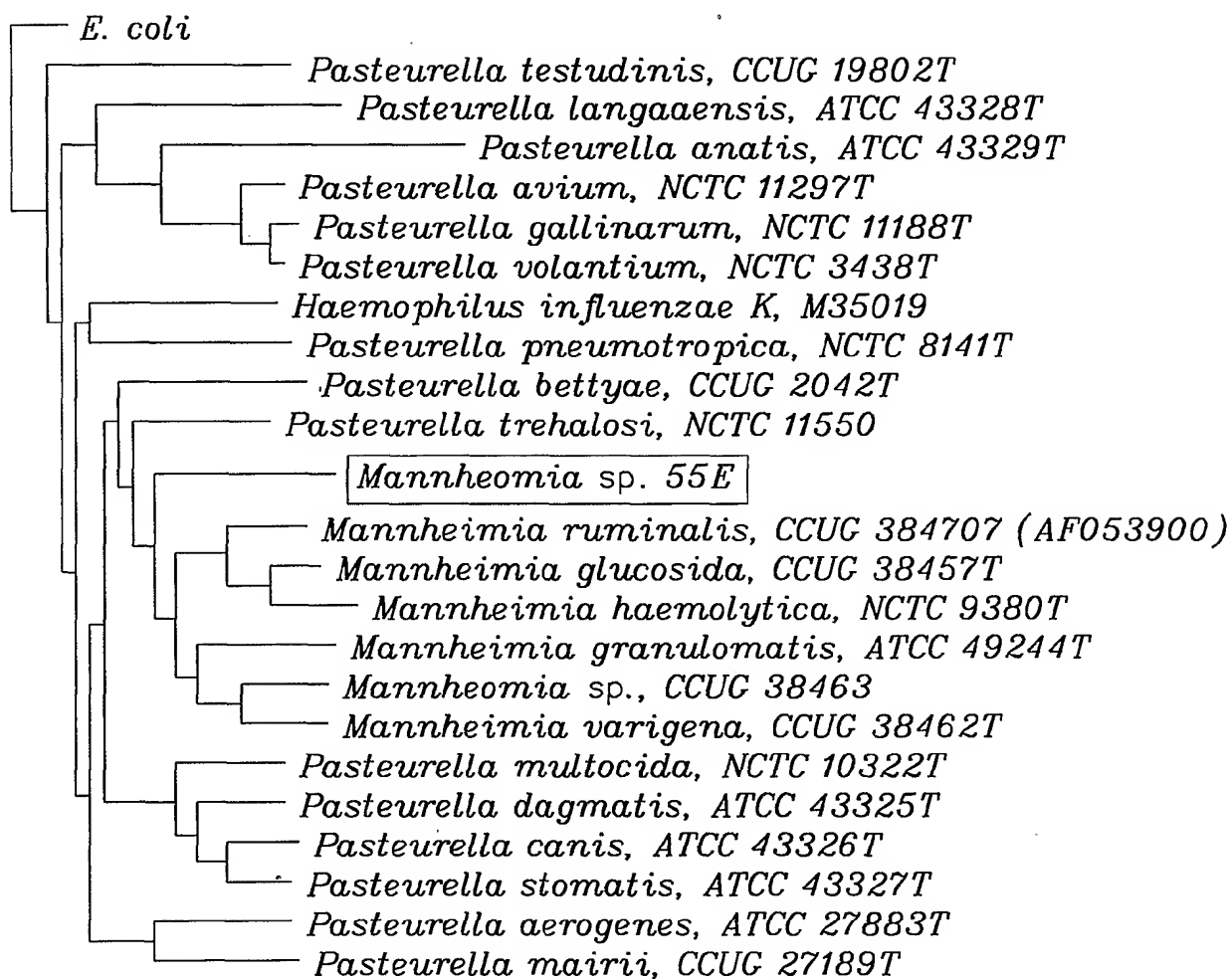
15

9. The process of claim 7, wherein the incubation is carried out at a temperature of 35-45°C at a pH of 6.0-7.5 with a supply of oxygen at a flow rate of 0.5-1.0 vvm.

20 10. A process for producing lactic acid, the process comprising:

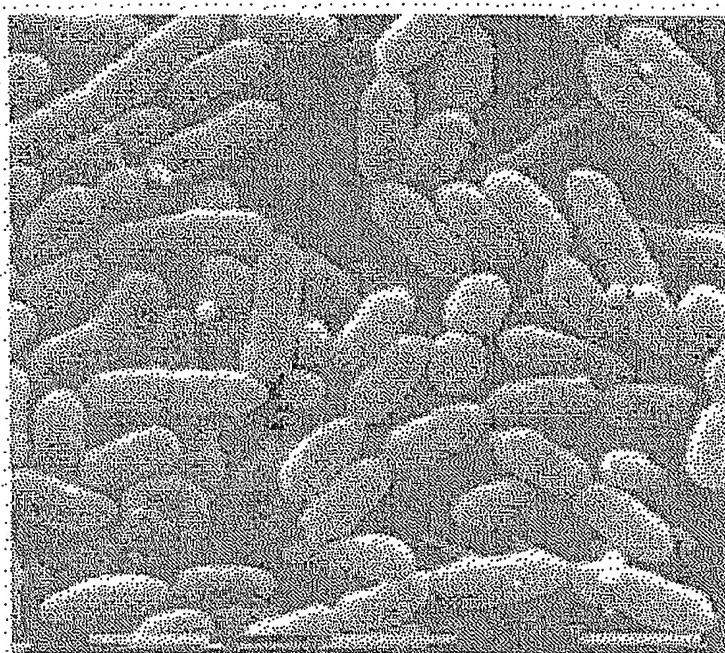
incubating *Mannheimia* sp. 55E with Accession Number KCTC 0769BP of claim 1 in a medium containing 5-60 g/L of glucose at a temperature of 35-45°C at a pH of 6.0-7.5 with a supply of oxygen at a
25 flow rate of 0.5-1.0 vvm; and
obtaining lactic acid from the medium.

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FIG. 1

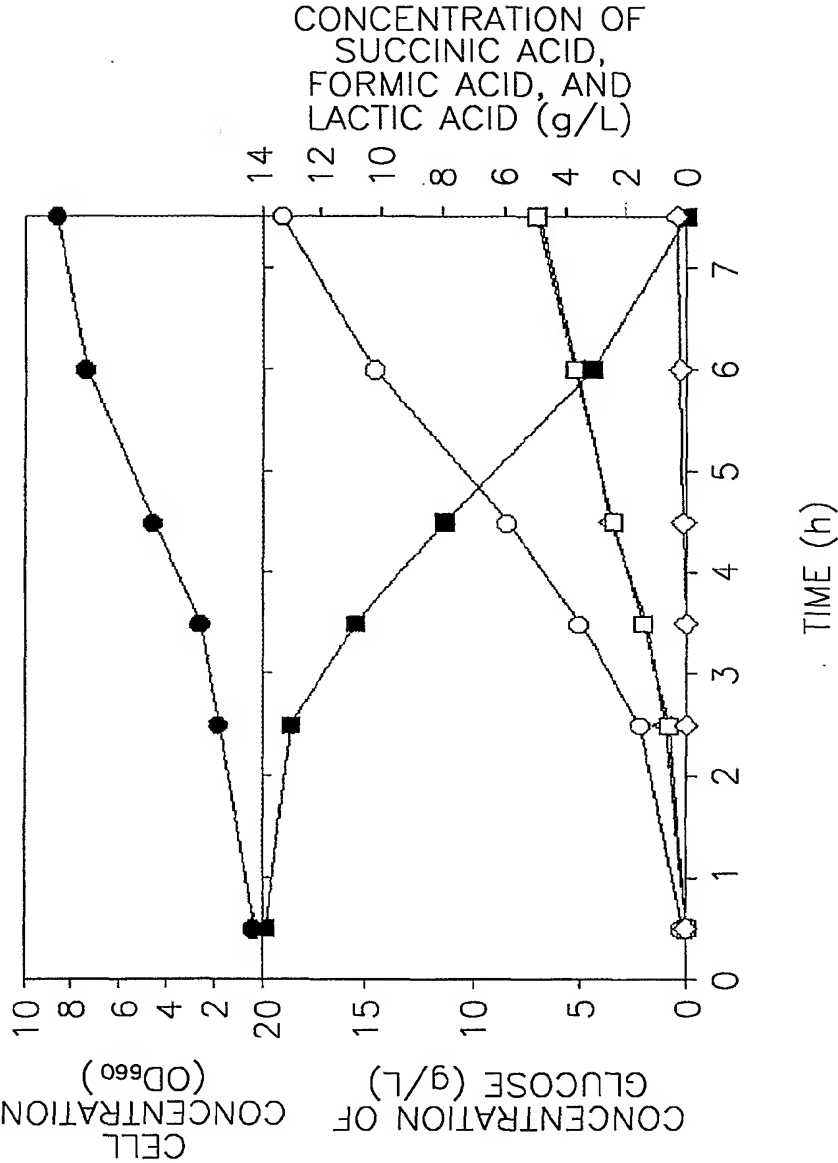
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FIG. 2



3/5

FIG. 3



4/5

FIG. 4

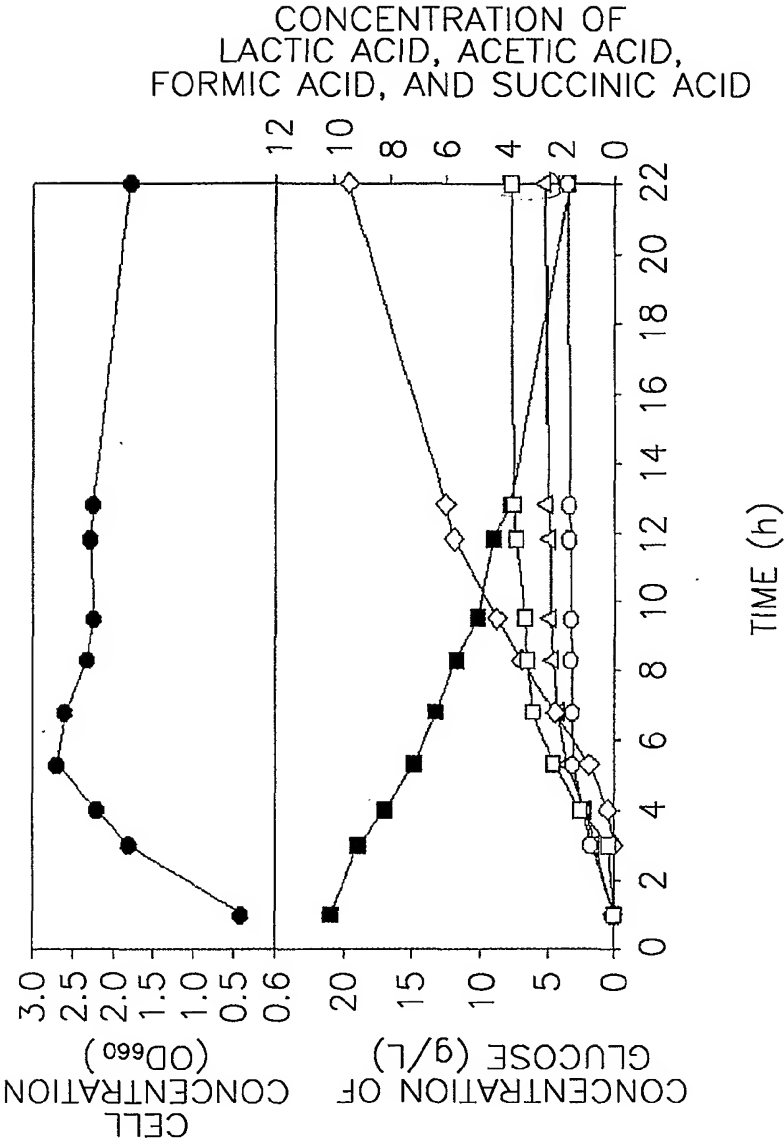
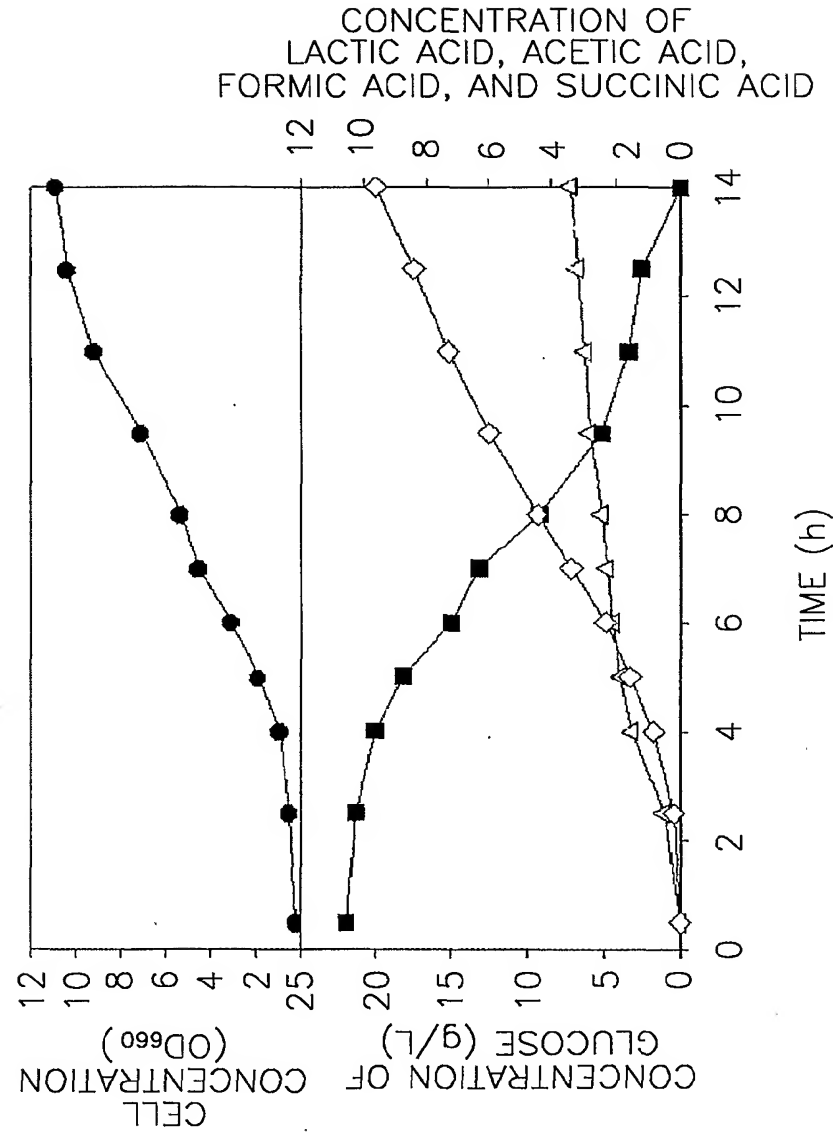


FIG. 5



SEQUENCE LISTING

<110> Korea Advanced Institute of Science and Technology

<120> Microorganism Capable of Producing Organic Acids and Process for Producing Organic Acids using the Microorganism

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<212> DNA

<213> Artificial Sequence

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<400> 1

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20

<210> 2

<211> 17

<212> DNA

<213> Artificial Sequence

<220>

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<400> 2

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